Identification of *Vibrio cholerae* and Detection of *omp*W Gene in Isolates of Raw Vegetables from Kuching, Sarawak

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This project is submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honours

(Resource Biotechnology Programme)

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

2010

ACKNOWLEDGEMENT

Special thanks to all contributors who were involved in my Final Year Project (FYP). Firstly, thanks to Dr. Lesley Maurice Bilung (supervisor) and Prof Dr Ismail Ahmad (co-supervisor), who successfully guided and assisted me in completing my FYP. A very special appreciation to Microbiology Lab postgraduate students (Kho Kai Ling, Chen Yik Ming, and Mr Adom) because patiently taught and instructed me on various technical aspects of my project. I really appreciate the knowledge I have received and will attempt to practice what I have learned in future. To all my friends who were directly and indirectly involved in my project, thank you so much for all the assistance and guidance. Thank be to God for His fully blessing from the beginning of my project until completion. Last but not least, my greatest appreciation to my beloved parents and family members, as well as brothers and sisters in Christ for all your love and care since the inception of my final year project.

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LIST OF ABBREVIATIONS

ACE	Accessory Cholera Enterotoxin
AGE	Agarose Gel Electrophoresis
B7	Kota Sentosa
bp	Base pair
C	Cucumber (*Cucumis sativus*)
CT	Cholera Toxin
DNA	Deoxyribonucleic acid
g	Gram
H_2S	Hydrogen sulphide
IM	Inner membrane
K	Kangkong or water spinach (*Ipomaea aquatica*)
LB	Luria Bertani
MRVP	Methyl Red – Voges-Proskauer
ml	Milliliter
mm	Millimeter
NA	Nutrient Agar
NaCl	Sodium chloride
n. d.	No date
OM	Outer membrane
OMP	Outer Membrane Protein
*omp*W	Outer membrane protein W gene
PCR	Polymerase Chain Reaction
S	Stutong
SC	Simmons citrate
SCF	Scientific Committee on Food
SI	Samar Indah
SIM	Sulphide, Indole and Motility
sp./spp.	Species
T	Tomato (*Lycopersicon esculentum*)
TBE	Tris-Borate-Ethylene di-amine tetra-acetic Acid
TCBS	Thiosulfate Citrate Bile Sucrose
TCP	Toxin-Coregulated Pilus
TSI	Triple Sugar Iron
VP	Voges-Proskauer
V. cholerae	*Vibrio cholerae*
V. parahaemolyticus	*Vibrio parahaemolyticus*
WHO	World Health Organization
ZOT	Zonula Occludens Toxin
^oC	Degree Celsius
%	Percent
µl	Micro liter

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ABSTRACT

Vibrio cholerae abundantly exist in aquatic sources. Ingestion of pathogenic *V. cholerae* O1 and O139 which generally found in raw and undercooked seafood can cause severe and sometimes lethal diarrheal disease called cholera. Therefore, an appropriate evaluation of existence of *V. cholerae* strain in selected *V. cholerae* isolates from raw vegetable samples in Kuching area is important. This study was attempted to detect *omp*W gene which is a virulence factors of *V. cholerae*. A total of 50 *V. cholerae* isolates were enriched in Luria Bertani (LB) broth and the isolation were performed via Thiosulfate Citrate Bile Sucrose (TCBS) agar. Then, forty presumptive *V. cholerae* isolates were subjected to morphological and biochemical tests. Furthermore, Polymerase Chain Reaction (PCR) amplification was performed specifically for *omp*W gene detection. Among 40 *V. cholerae* isolates, there was no presence of *omp*W gene. PCR results supported by biochemical test results indicate that all the isolates are not *V. cholerae*. This study suggests a low occurrence of *V. cholerae* in raw vegetables.

Keywords: *Vibrio cholerae*; *omp*W gene; Polymerase Chain Reaction (PCR); Thiosulfate Citrate Bile Sucrose (TCBS) agar; biochemical tests.

ABSTRAK

Vibrio cholerae banyak terdapat di dalam sumber akuatik. Pengambilan makanan laut yang mentah atau separuh masak boleh menyebabkan penyakit cholera yang sederhana dan kadang-kadang boleh membawa maut kerana kemungkinan mengandungi V. cholerae O1 dan O139 yang boleh membawa penyakit. Oleh yang demikian, satu penilaian tentang kewujudan V. cholerae di dalam sampel sayur-sayuran mentah di kawasan Kuching adalah penting. Penyelidikan ini berusaha untuk mengenalpasti kewujudan gen ompW yang merupakan salah satu daripada faktor berbahaya di dalam V. cholerae. Lima puluh V. cholerae isolet telah diperkayakan di dalam larutan LB serta diasingkan di dalam agar TCBS. Kemudian ujian morfologi dan ujian biokimia dijalankan untuk 40 isolet pra-pengesahan. Seterusnya, PCR dilakukan untuk mengenalpasti gen ompW dan didapati bahawa gen ompW tidak terdapat di dalam kesemua 40 V. cholerae isolet. Keputusan PCR disokong oleh keputusan ujian biokimia yang menunjukan semua isolet adalah bukan V. cholerae. Kajian ini jelas menunjukkan bahawa kewujudan V. cholerae di dalam sayur-sayuran mentah adalah rendah.

Kata kunci: Vibrio cholerae; ompW gene; PCR; TCBS agar; ujian-ujian biokimia.

CHAPTER 1

INTRODUCTION AND OBJECTIVES

1.1. Introduction

Cholera is one of the most common gastrointestinal diseases affecting human communities around the world. It is caused by pathogenic *Vibrio cholerae* belonging to the O1 and O139 serogroups. Cholera become endemic in Asia and Africa over the years can be severe-mild and sometimes lethal diarrheal illness. *V. cholerae* is a gram-negative bacterium that is facultatively anaerobic, comma shape, salt-tolerant (2 to 4 % NaCl), oxidase-positive, and capable of fermentative metabolism. *Vibrio* species taxonomically has a close relationship with genera *Aeromonas* and *Plesiomonas* (Faruque *et al*., 1998). *V. cholerae* O1 and O139 carried virulence factors that cause them to be pathogenic to human such as cholera toxin (CT), zonula occludens toxin (ZOT), accessory cholera enterotoxin (ACE), toxin-coregulated pilus (TCP) as well as outer membrane protein (OMP). Outer membrane in Gram-negative pathogenic bacteria plays an important function in the infection and pathogenicity to host (Tsolis, 2002). *omp*W is species-specific gene for *V. cholerae* species plays major roles in bacterial infections and host immune response (Qian *et al*., 2007).

The organism is frequently present in watery environment as well as in association with aquatic animal (Todar, 2009). Therefore, they are usually detected and identified in seafood such as cockle and shrimp. Raw fruits and vegetables also have been known as sources of human diseases especially gastrointestinal illness (Beuchat, n. d.). Fruits and vegetables carry a natural non-pathogenic epiphytic microflora, however, they can be contaminated with pathogens from humans or animals sources during growth and further

handling processes (Scientific Committee on Food (SCF), 2002). SCF (2002) mentioned that fresh produce had been proved involve in many outbreaks of foodborne disease particularly in Europe, Japan, United States, and Canada.

Rapid detection of *V. cholerae* is usually conducted via biochemical testing and PCR amplification. Sometimes, the results obtained from biochemical tests were problematic and can be corrected and compared with PCR amplification result for more accurate and valid research or study. Many studies conducted have proven the presence of virulence factors in pathogenic *V. cholerae*. A recent study conducted by Chatterjee *et al*. (2009) investigated the incidence of virulence factors and clonality among clinical strains of non-O1 and non-O139 *V. cholerae* isolates from hospitalized diarrheal patients in Kolkata, India. The study detected *V. cholerae* strains in 197 stool specimens where 135 of the specimens contain *V. cholerae* strains of serogroup O1, 2 specimens contained strains of serogroup O139, and 60 strains belonged to non-O1 and non-O139 serogroups. The results indicate the prevalence of pathogenic *V. cholerae* in hospitalized diarrheal patients is increasing. Additionally, Kanungo *et al.* (2010) also reported the analysis of cholera in India in 1997-2006 and they found that in the 10 years period, cholera occurs over a wider geographic area in India than previously recognized.

Outbreaks of cholera in Malaysia have been reported in many states. Petaling District Health Office Annual Report 2000 reported that there were 82 cases of cholera have been detected in the Petaling district for the past five years (Rushidi *et al*., 2002). According to Rushidi *et al.* (2002), at the end of June 2001, there was a cholera outbreak reported from the Midlands Estate Petaling which gave rise to three cases and fifteen carriers identified. In 2002, Radu *et al*. (2002) molecularly characterized the *V. cholerae* O1 outbreak strains in Miri,

Sarawak and their result showed the presence of core toxin region among the isolates, and confirmed the co-occurrence of the *tcp*, *ctx*, *zot*, and *ace* in 32 of the 33 *V. cholerae* O1 clinical isolates. They proved that the area was affected by pathogenic *V. cholerae* O1 strains.

Recently, in 2009, Teh *et al*. performed the molecular characterization of serogrouping and virulence genes of Malaysian *V. cholerae* isolated from clinical and environmental sources as well as from water and seafood samples. According to their multiplex PCR results, among the panel of 29 Malaysian strains tested, 1 was subtyped as O139 serogoup, 7 were non-O1/non-O139, and 21 were subtyped as O1 serogoups, with 20 strains expressing toxigenic genes *tox*R, *omp*W, *hly*A, *ctx*A, and *tcpl* (Teh *et al*., 2009). Their result obviously showed that the occurrence of pathogenic *V. cholerae* in Malaysia is high. Therefore, this study is important to detect the presence of pathogenic *V. cholerae* in food sources specifically on raw vegetables sold in Kuching and Samarahan areas.

1.2. Objectives

This study is conducted to detect the presence *omp*W gene in *V. cholerae* isolates from selected raw vegetables in selected area in Kuching, Sarawak. There are three types of raw vegetables chosen, cucumber (*Cucumis sativus*), kangkong or water spinach (*Ipomaea aquatica*), and tomato (*Lycopersicon esculentum*), collected from Stutong, Samar Indah and Kota Sentosa, Kuching, Sarawak. Isolation and identification of *V. cholerae* isolates using selective medium and biochemical test were conducted first to determine the *V. cholerae* species. Subsequently, Polymerase Chain Reaction (PCR) was performed to detect *omp*W gene in the isolates using species-specific *omp*W primer according to Nandi *et al*. (2000). The objectives of the study are:

1. to determine the occurrence of *Vibrio cholerae* in selected raw vegetables isolates collected from selected area in Kuching.
2. to isolate and identify the presence of *Vibrio cholerae* species in raw vegetable isolates (kangkong or water spinach, cucumber, and tomato) from Stutong, Samar Indah, and Kota Sentosa using selective agar and series of biochemical tests.
3. to determine the presence of *omp*W gene in *Vibrio cholerae* isolates using Polymerase Chain Reaction (PCR) amplification.

CHAPTER 2

LITERATURE REVIEWS

2.1. *Vibrio cholerae*

2.1.1. Characteristics and taxonomy

V. cholerae is divided into more than 130 O serogoups but so far only organisms of the O1 serogroups are associated with cholera in humans (Manning *et al*., 1994). According to Manning *et al*. (1994), the O1 serotype exists as two biotypes which are classical and E1 Tor. Furthermore, *V. cholerae* O1 strains of both biotypes have been subdivided into three serotypes namely Inaba, Ogawa, and Hikojima (Manning *et al*., 1994). *V. cholerae* is a member of the family *Vibrionaceae*, is facultatively anaerobic, gram negative, non-spore-forming, curved rod shape about 1.4-2.6 µm long, and capable of respiratory and fermentative metabolism (Anon., n. d.). *V. cholerae* O1 and O139 serogroups were recognized as agent of diarrheal disease known as cholera (Srisuk *et al.*, 2009). According to Chen *et al*. (2003), *V. cholerae* strains O1 and O139 serotypes almost always carry the *ctx* genes which O serotype is always used as a marker for the pathogenic strains. *Vibrionaceae*, *Pseudomonadaceae* and *Enterobacteriaceae* are all grouped in the *Gammaproteobacteria* but vibrios are differentiated from other enteric species by being oxidase-positive and motile (Todar, 2009). Taxonomic studies of related organisms have pointed out a close relationship among three genera known as *Vibrio, Aeromonas*, and *Plesiomonas* (Faruque *et al*., 1998).

2.1.2. Habitat

V. cholerae can be isolated in abundance from aquatic or estuarine sources (Nandi *et al*., 2000). Todar (2009) mentioned that *Vibrios* are the most common organisms in water surface and often occur in both marine and freshwater habitats and in associations with aquatic animals.

2.1.3. The disease: Cholera

V. cholerae is the causative agent of the severe and sometimes lethal diarrheal disease cholera (Hochhut *et al*., 2001). Strains of *V. cholerae* that carry *ctx* genes can produce cholera toxin and known as toxigenic strains responsible for cholera epidemics (Chen *et al*., 2004). Panicker *et al*., (2004) in their study mentioned that *V. cholerae*-associated illnesses in the United States are from the consumption of raw or undercooked shellfish. Furthermore, Govea *et al*., (2001) described cholera as an acute intestinal disease with watery diarrhea, vomiting, high dehydration, acidosis, and circulation disorders caused by *V. cholerae*. Cholera is caused by *V. cholerae* serogroup O1 (Chow *et al*., 2001). Reller *et al.* (2001) reported that the duration range of illness was between 1-7 days with symptoms included diarrhea, vomiting, and leg cramps.

2.2. *omp*W (outer membrane protein) gene

Cell wall of Gram negative bacteria consists of three important layers on the surface envelope which are the cytoplasmic or inner membrane (IM), the outer membrane (OM), and the periplasmic space between IM and OM (Nandi *et al.*, 2005). The OM acts as protective barrier that hinders the permeability of both hydrophobic and hydrophilic compounds due to the presence of lipopolysaccharide (Hong *et al*., 2006). According to Lin and Zhang (2002), major

components of OM are phospholipids, lipolysaccharide, and protein which serve as a physical barrier between the bacterial body and its surrounding and make the organism resistant to host defense factors and toxic materials such as bile salts and antibiotics. OM mass consists of proteins either in the form of integral membrane proteins or lipoproteins, which contains considerable number of β-pleated sheet in the form of a barrel that enables the organism to maintain its structural integrity as well as allow selective permeability of solutes across the membrane (Koebnik *et al.*, 2000).

In *V. cholerae*, which belong to OmpW/AlkL family, the putative gene has been described by Jalajkumari and Manning (1990) as a 22 kDa molecule. The gene was present in the chromosome II of *V. cholerae* (Heidelberg *et al*., 2000). Expression of *omp*W gene in *V. cholerae* was extensively studied among scientists. In 2000, Nandi *et al*. in their study for species-specific identification of *V. cholerae* using primers targeted to *omp*W gene, mentioned that the *omp*W primers showed 100% specificity for all tested *V. cholerae* strains and the result suggested the method is highly suitable as genetic marker for the organism. Recently, Tamrakar *et al.* (2009) characterized *V. cholerae* from deep ground water in a cholera endemic area at Central India and found that all the isolates were PCR positive for *omp*W gene which confirmed their biochemical results.

2.3. Epidemiology of cholera

V. cholerae is an important cause of diarrheal disease in many parts of Asia and Africa (Chow *et al.*, 2001). In 2001, Chow *et al.* tried to detect RTX (repeat in toxin) toxin gene that was proven as important virulence factors among gram-negative bacteria in *V. cholerae* by highly specific PCR amplification. They analyzed more than 100 clinical and environmental isolates

of *V. cholerae* which collected from 1986 to 1999 in five different countries, Hong Kong, China, Singapore, Thailand, and Ukraine. The result from their study showed that all the isolates were proven toxigenic by both PCR and cytotoxicity assay. In 2003, Chen *et al.* successfully isolated 97 strains of *V. cholerae* from various seafoods in Malaysia in 1998 to 1999 and 20 strains were detected carrying *ctx* gene and produced cholera toxin responsible for cholera epidemics.

2.4. Transmission and treatment of cholera

Transmission of *V. cholerae* is associated with consumption of contaminated water and contaminated foods (Miyazato *et al*., 2004). Mintz *et al*., (1994) mentioned that survival and growth of *V. cholerae* O1 in food were enhanced by low temperatures, high organic content, near-neutral pH, high moisture, and absence of competing flora. According to Epidemiology Unit of Ministry of Healthcare and Nutrition in Colombo, commonly used treatment of cholera is replacement of lost fluids and salts. They recommended that the use of oral rehydration salts (ORS) is the fastest and efficient way of treatment where most patients will recover in three to six days. Besides, patient may be treated with antibiotics such as tetracycline and erythromycin (Epidemiology Unit of Ministry of Healthcare and Nutrition, n. d.). Treatment using antibiotic or antimicrobial therapy can be a selective way for cholera treatment because it can reduce the volume of diarrhea as well as shorten the period of symptoms and the removal of *Vibrios* in the stool (Miyazato *et al*., 2004).

2.5. Pathogenicity of *Vibrio cholera*

Virulence factors that are normally found in epidemic *V. cholerae* O1 strain are cholera toxin (CT), the colonization factor toxin-coregulated pilus (TCP), and the regulatory protein ToxR which contribute to pathogenic characteristic of *V. cholerae* (Pichel *et al.*, 2002). According to World Health Organization (WHO) guidelines, both *V. cholerae* O1 and O139 are now proven causes of cholera while non-O1 and non-O139 *V. cholerae* can cause illness but they do not show the public health effect of the O1 and O139 serogroups.

2.6. Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar

TCBS is a selective medium for isolation of *V. cholerae*. It removes most non target bacteria in clinical samples but it is not recommended for environmental samples because many bacteria able to survive in natural water sources can produce colonies in TCBS and their appearance similar to *V. cholerae* (Choopun *et al*., 2001). According to Baron *et al*., (2007) TCBS is commonly suggested for isolation of *Vibrio* spp. because it eliminates non-bile salt tolerant species and able to isolate vibrios from their natural estuarine environment. The fermentation of sucrose by *V. cholerae* produces yellow colonies due to a change in pH (Wright, 2008). TCBS is available commercially, does not require autoclaving, is highly selective, and contains sucrose which allows the differentiation of *V. cholerae* and *V. parahaemolyticus* (Kay *et al*., 1994). According to Kay *et al*., (1994) overnight growth of *V. cholerae*, usually 18-24 hours, on TCBS will produce large (2-4 mm in diameter), slightly flattened, yellow colonies with opaque centers and partially transparent peripheries.

2.7. Identification of *Vibrio cholerae*

Identification of *V. cholerae* usually achieved through a series of biochemical tests after isolation of bacterium on selective media. Problems encountered in biochemical identification of bacteria can be overcomed by targeting species-specific genes with techniques such as PCR. According to Faruque *et al.* (1998), *V. cholerae* is well detected using a series of biochemical tests and DNA homology studies.

2.8. Polymerase Chain Reaction (PCR)

Erlich (1992) defined PCR as an *in vitro* method for the enzymatic synthesis of specific DNA sequences using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. According to Koch *et al*., (2001), PCR was introduced by Mullis *et al*. in 1985 that involves denaturation of double-stranded DNA to provide single-stranded templates to which specific oligonucleotide primers are hybridized, continued by primer extension with a thermostable DNA polymerase. Detection of pathogens by PCR has been shown to be highly specific and relatively less time-consuming than conventional methods (Panicker *et al*., 2004). According to Panicker *et al*., (2004) the conventional methods mentioned include most-probable-number (MPN) technique in association with biochemical tests or colony blot hybridization with gene-specific probes which are time-consuming and labor intensive. Nowadays PCR is being increasingly used for direct detection of microorganisms, detecting genes coding for virulence factors and determining the presence of genes responsible for anti-microbial resistance (Aygan, 2005). Aygan (2005) mentioned that PCR is based on three steps of DNA synthesis reaction: (1) denaturation of the template into

single strands; (2) annealing of primers to each original strand for new strand synthesis; and (3) extension of the new strands from the primers.

Majority of the studies related to *V. cholerae* involved PCR amplification. Faruque *et al*. (2002) investigated the emergence and evaluation of *V. cholerae* O139 by using PCR-based analysis. Rivera *et al.* (2001) conducted multiplex PCR for examination and detection of cholera-related genes in environmental isolates of *V. cholerae*. Recently, Lei *et al.* (2008) applied multiplex PCR method in their study of detection of six common foodborne pathogens included *V. cholerae*. This indicates the significance of PCR amplification technique for endemic disease studies.

CHAPTER 3

MATERIALS AND METHODS

3.1. Sources of *Vibrio cholerae* isolates and enrichment

Isolates of *V. cholerae* were supplied by post graduate student. Isolates were collected from raw vegetable samples. Three types of raw vegetables were selected which are tomato (*Lycopersicon esculentum*), cucumber (*Cucumis sativus*), and kangkong or water spinach (*Ipomaea aquatica*). The samplings were conducted within different weeks and hours from selected area in Kuching which are Stutong, Kota Sentosa, and Samar Indah. Fifty raw vegetables cultures for *V. cholerae* were enriched and grown overnight in Luria Bertani (LB) broth at 37°C in 150 rpm shaking incubator.

3.2. Isolation of *Vibrio cholerae* isolates via selective medium

Isolation of *V. cholerae* isolates were performed using selective medium, Thiosulfate Citrate Bile Sucrose (TCBS) agar. A loop full of each enriched culture was streaked onto TCBS plate. Then, the plate was incubated overnight at 37°C. The growth of yellowish colonies on TCBS agar are assumed as the growth of *Vibrio* related species. The yellowish colonies that grow on TCBS plate were subjected to biochemical testing and gram staining for identification and confirmation of *V. cholerae* isolates. There are seven types of biochemical tests were performed namely methyl-red, Vogues-Proskauer, sulphide, indole and motility (SIM), Simmons citrate, oxidase, salt tolerance, and triple sugar iron (TSI) tests. Preparation of stock culture and working culture were done before proceeding to biochemical testing.

3.3. Identification of *Vibrio cholerae* via gram staining and biochemical tests

For each isolate, individual colony from an overnight culture on nutrient agar (NA) plate was sub-cultured to perform gram staining and each biochemical test. Procedures for gram staining and biochemical test were conducted and interpreted according to Bergey's Manual of Systemic Bacteriology (Hons & Krieg, 1984).

3.3.1. Gram staining

Gram staining is always performed before identification of bacteria species. If red or pink color and comma shape of organism was observed under 1000X light microscope, it indicates presumptive of *V. cholerae* species. First, a drop of distilled water was added on a slide and individual colony of bacteria was emulsified on it and then allowed to dry. Next, the staining and subsequent steps were done according to Figure 3.1.

3.3.2. Methyl red – Vogues-Proskauer (MRVP) test

Two of the biochemical tests were performed which are methyl red test and Vogues Proskauer test. Individual bacterial colony was inoculated into single universal bottle containing MRVP media and incubated for 48 hours at 37°C. After 48 hours, about half of the culture was transferred to a clean universal bottle. One universal bottle of culture was used to conduct MR test, the second bottle used for VP test. For MR test, methyl red was added and formation of red color indicates a positive result. For VP test, first Barritt's reagent A (alpha-naphtol) was added and then Barritt's reagent B (potassium hyroxide). The culture was allowed to sit for 15 minutes for color formation to occur. Formation of red color indicates a positive result. The result was recorded.

3.3.3. Sulphide, indole, and motility (SIM) test

Only indole production was tested and observed in this test. Individual bacterial colony was inoculated into SIM media by stabbing the agar. The tube was incubated for 24 hours at 37°C. After 24 hours, the tube was removed from incubator and a drop of Kovac's reagent was added to the media to detect the indole production by bacteria. The appearance of a red or pink layer on the top of the media is a positive result. The result was recorded.

3.3.4. Simmons citrate (SC) test

Single bacterial colony was streaked on Simmons citrate slant agar in screw-capped tube. Then, the tube was incubated for 24 hours at 37°C. After 24 hours, the observation was made and recorded. The changes of media from green to bright blue color was considered as positive reaction.

3.3.5. Oxidase test

A drop of oxidase reagent was applied on filter paper. Then, single bacterial colony was picked up using a sterile toothpick and streaked on filter paper saturated with oxidase reagent. Immediate appearance of a dark purple color was observed which constitute a positive reaction and result was recorded.

3.3.6. Salt tolerance test

Individual colony was inoculated into NA broth with different sodium chloride (NaCl) concentration, 0 % NaCl and 8 % NaCl. Then, the inoculated broth incubated for 24 hours with shaking at 37°C. After 24 hours, the turbidity of the broth was observed and result was recorded. The development of turbidity of broth is considered a positive reaction.

3.3.7. Triple Sugar Iron (TSI) test

Individual colonies were inoculated into TSI media by stabbing the butt and streaking slant. Each tube was loosely capped, then incubated for 24 hours at 37°C. After 24 hours, the results were interpreted by recording the appearance of the TSI slants and butts which tests for hydrogen sulphide (H_2S) production, glucose fermentation, and utilization of sucrose or lactose.

3.4. Genomic DNA extraction via boiled cell extraction method

Genomic DNA extraction of *V. cholerae* was performed using boiled cell extraction method according to Bilung *et al.* (2005). Individual colony of *V. cholerae* from Nutrient Agar (NA) plate was inoculated into LB broth and grown overnight at 37°C in 150 rpm shaking incubator. 500 ml of cell culture were transferred into 1 ml Eppendorf tube and centrifuged at 10000 rpm for 5 min. The supernatant was discarded and the cell pellet was resuspended in 500 ml sterile distilled water. Then, the tube was centrifuged at 10000 rpm for 5 min and supernatant discarded before the cell pellet resuspended in 500 ml sterile distilled water. The tube was vortexed for few seconds before boiled for 10 minutes to facilitate cell lysis. After boiling for 10 minutes, the tube was immediately placed in ice for 10 minutes. Finally, the cell lysate was centrifuged and the clear supernatant was transferred to a new tube which will be used as DNA template in PCR amplification.

3.5. Polymerase Chain Reaction (PCR) amplification

PCR assay for detection of outer membrane protein W (*omp*W) genes was done according to procedure described by Nandi *et al.* (2000) with few modifications. The reaction mixture was subjected to an amplification of 30 cycles. Table 3.1, 3.2, and 3.3 showed the ingredients of reaction mixture, amplification steps, and primer sequence that were used. Negative control containing PCR reagent without DNA template was carried out for each PCR run to detect possible contamination in the reaction mixtures.

3.6. Agarose Gel Electrophoresis (AGE)

0.6 g of agarose powder and 50 ml of Tris-Borate-Ethylene di-amine tetra-acetic Acid (TBE) buffer were prepared to produce 1.2 % agarose solution. Then, the agarose solution was boiled in microwave until it was dissolved. The dissolved agarose was allowed to cool before being poured into gel tray. After the agarose gel has completely solidified, it was transferred into AGE box containing TBE buffer. Diluted DNA ladder (5 µl) and each PCR product (1 µl 6X loading dye plus 5 µl PCR product) were loaded into appropriate well in AGE box after well mixed. The loaded gel was run for 40 minutes at 90 volts. After 40 minutes, the gel was stained for 20 minutes in ethidium bromide solution and then exposed to ultraviolet (UV) light box to visualize the gel and detect the presence or absence of *omp*W gene. The good picture of gel was saved and labeled.

CHAPTER 4

RESULTS

I. Result for biochemical tests is summarized by Table 4.1.

Table 4.1: Results for biochemical tests

No.	Sample	Biochemical test							
		MR	VP	TSI	SC	Ox	In	Salt tolerance	
								0 % NaCl	8 % NaCl
1	S/S1/W3/6H/T	--	--	A/A,G	+ (S)	--	--	+	--
2	S/S2/W4/24H/C	--	+	K/A,G	+ (S)	--	--	+	--
3	S/S3/W5/6H/T	--	+	A/A,G	+ (S)	--	--	+	--
4	S/S3/W3/24H/C	--	+	K/A,G	+ (S)	--	--	+	--
5	S/S1/W4/24H/C	--	+	K/A,G	+	--	--	+	--
6	S/S1/W5/6H/C	--	--	A/A,G	+ (S)	--	--	+	--
7	S/S2/W3/6H/C	--	--	A/A,G	+	--	--	+	--
8	S/S2/W4/24H/K	--	+	K/A,G	+	--	--	+	--
9	S/S1/W4/24H/T	--	+	K/A,G	+	--	--	+	+
10	S/S2/W4/24H/T	--	+	K/A,G	+	--	--	+	--
11	S/S2/W3/6H/T	--	--	K/A,G	+ (S)	--	--	+	--
12	B7/S2/W5/6H/K	--	+	K/A,G	+ (S)	--	--	+	--
13	B7/S2/W4/24H/K	+	--	K/A,G	+ (S)	--	--	+	--
14	B7/S1/W2/24H/C	+	+	A/A,G	+	--	--	+	+
15	B7/S3/W4/24H/C	--	+	A/A,G	+ (S)	--	--	+	--
16	B7/S1/W5/6H/K	--	+	K/A,G	+ (S)	--	--	+	--
17	B7/S3/W5/6H/T	--	+	A/A,G	+ (S)	--	--	+	--
18	B7/S3/W5/24H/C	--	+	K/A,G	+ (S)	--	--	+	--
19	B7/S3/W5/24H/T	--	+	K/A,G	+ (S)	--	--	+	--
20	B7/S3/W4/24H/T	--	--	K/A,G	+	--	--	+	--
21	B7/S1/W3/6H/T	--	--	K/A,G	+ (S)	--	--	+	--
22	B7/S2/W3/24H/T	--	--	A/A,G	+ (S)	--	--	+	--
23	SI/S1/W2/24H/C	--	--	A/A,G	+ (S)	--	--	+	--
24	B7/S1/W2/24H/T	--	--	K/A,G	+ (S)	--	--	+	--
25	SI/S3/W3/6H/C	--	+	K/A,G	+	--	--	+	--
26	SI/S3/W3/24H/K	--	+	K/A,G	+	--	--	+	--
27	SI/S3/W4/6H/T	--	--	A/A,G	+	--	--	+	--
28	S/S3/W4/6H/K	--	+	K/A,G	+	--	--	+	+
29	SI/S3/W2/6H/T	--	+	K/A,G	+ (S)	--	--	+	--
30	SI/S3/W5/6H/K	--	--	K/A,G	+ (S)	--	--	+	--
31	SI/S1/W2/6H/C	--	+	K/A,G	+ (S)	--	--	+	--